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The neurotransmitters gamma aminobutyric acid (GABA) and nitric oxide (NO) play a role in maintaining the larval state in the eastern mud snail, *Nassarius obsoletus*, but how GABAergic and nitrergic neurons are organized in the neural circuit that controls metamorphosis is unknown. Because of previous work by other members of my laboratory, I hypothesized that GABAergic neurons would be upstream from the nitrergic ones. Larval *N. obsoletus* were exposed to various combinations of GABAergic and nitrergic compounds in bath application and the resulting numbers of metamorphosed individuals were counted after 24 and 48 hours. I performed multiple sets of experiments and while some indicated that GABAergic neurons were upstream from nitrergic ones, the data as a whole did not reveal a clear organizational scheme to this pathway. Thus, I suggest instead that GABA and NO may function in separate pathways to influence the onset of metamorphosis.

# WHERE DO NITERGIC AND GABAERGIC NEURONS LIE IN THE METAMORPHIC PATHWAY OF *NASSARIUS OBSOLETUS*?

by

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# CHAPTER I

# INTRODUCTION

Metamorphosis is defined as a transition between two distinct life history stages. The metamorphic phenomenon is perhaps most commonly associated with insects and amphibians, but it also extends to molluscs. In general, larval and adult stages often occupy different ecological niches, feed on different food sources, and differ morphologically, behaviourally, and physiologically (Chia and Koss, 1988). The adults of many gastropod species occupy rocky intertidal areas or mudflats and can be omnivorous, carnivorous, or herbivorous. Conversely, larvae are generally planktonic and, if they feed, ingest unicellular algae. Larvae of metamorphic molluscs develop rudiments of most of their adult organs, which are overlain with antecedental larval structures and organs. These larval features are all lost when the larvae transition to the juvenile state (Scheltema, 1962; Lin and Leise, 1996; Gifondorwa and Leise, 2006; Ruiz-Jones and Hadfield, 2011).

## ***Nassarius obsoletus*, Life History, and Behavior**

The Eastern mud snail, *Nassarius obsoletus* (Bouchet and Rocroi, 2005), is a marine mollusc that has been used for decades as a model organism for the study of

development and metamorphosis (Leise and Cahoon, 2012; Collier, 2002). The benthic adult snail acts as a major agent of sediment disturbance on intertidal mudflats (Connor et al., 1982; Kelaher et al. 2003), lays egg capsules in the spring, and is an obligate omnivore (Leise et al. 2009) while its larvae are planktotrophic, feeding on single-celled algae (Scheltema, 1962). Larval metamorphosis in physiologically competent *N. obsoletus* can be induced by diatoms that occur in their natural environment (Leise et al., 2009) or by exogenous serotonin (5-HT) in bath and injection applications (Levantine and Bonar, 1986; Couper and Leise, 1996).

## Morphology and Neuroanatomy of Larval *N. obsoletus*

Upon hatching from egg capsules, larvae are approximately 250μm in shell length and the brain ganglia are still developing. That said, veliger larvae have eyes, tentacles, a functional gut and nervous system, rudiments of all adult ganglia and by metamorphosis a bifurcated propodium (Lin and Leise, 1996; Dickinson and Croll, 2003). The defining characteristic of the veliger larval stage is the presence of the ciliated velar lobes, large wing-like structures edged in motile cilia which the larvae use to swim and feed, that are lateral to the eye spots. The velar lobes are innervated by the apical ganglion (AG), a structure present in the larval nervous system that lies atop the cerebral commissure. The AG occurs in all members of the Mollusca that have been examined and may be responsible for driving many larval behaviours, such as swimming and feeding (Kempf et al., 1997; Marois and Carew, 1997; Pires et al., 2000; Ruiz-Jones and Hadfield, 2011).

Research done on the nudibranch *Phestilla sibogae* has implicated the AG in the detection of metamorphic inducers (Hadfield et al., 2000). In *N. obsoletus*, this ganglion has a total of 28 neurons, including all 3 basic types: sensory neurons, motor neurons and interneurons (Marois and Carew, 1997; Hadfield et al., 2000; Dickinson and Croll, 2003). In *P. sibogae*, the AG contains ciliated sensory neurons that are likely to transmit stimuli from the environment to the cerebral ganglia (Bonar and Hadfield, 1974). Studies on *P. sibogae* and *N. obsoletus* have demonstrated that after a metamorphic inducer is detected and metamorphosis has begun, the AG undergoes programmed cell death and is ultimately lost during this transition to the juvenile stage (Gifondorwa and Leise, 2006; Ruiz-Jones and Hadfield, 2011). In several gastropods, 5-HT is produced by a distinct and limited number of neurons in the AG (Kempf et al, 1997; Thavaradhara and Leise, 2001). In *N. obsoletus*, five of the twenty-eight neurons in the AG are serotonergic (Leise et al, 2004; Dickinson and Croll, 2003), while all twenty-eight appear to be nitrergic (Thavaradhara and Leise, 2001). Physiological studies in *N. obsoletus* have provided evidence that these neurotransmitters play important roles in metamorphosis (Couper and Leise, 1996; Froggett and Leise, 1999). Once the AG detects an appropriate metamorphic signal, larvae exhibit settlement behaviour and start alternating between swimming and crawling on the substratum, which can facilitate the metamorphic process (Hadfield and Koehl, 2004).

**Serotonin (5-HT) and *N. obsoletus***

Serotonin is a highly conserved monoamine neurotransmitter found in the nervous systems of all bilaterian species. It is perhaps most widely known for its role in controlling mood in the human brain (Meyer and Quenzer, 2005). In molluscs, 5-HT can increase the heart rate, increase ciliary beating of the gills, or induce spawning, depending on the tissue in which it is expressed (Meechonkit et al., 2010). It is synthesized from L-tryptophan by the enzymes tryptophan hydroxylase and aromatic L-amino acid decarboxylase and 5-HT is a metamorphic inducer in many, but not all, molluscan species (Leise and Cahoon, 2012). In *N. obsoletus,* 5 serotonergic neurons occur in the AG of competent larvae (Leise et al., 2004). Serotonin acts at both metabotropic and ionotropic receptors and, in our snails, the receptors of interest are likely to be the 5-$HT_{2B}$ and the 5-$HT_3$ receptors. 5-$HT_{2B}$ receptors are metabotropic and typically mediate many of the central nervous system effects of 5-HT, while the 5-$HT_3$ receptors are ligand-gated ion channels. Both of these types of receptors mediate excitatory post-synaptic responses. However, inhibitory receptors, such as the 5-$HT_{1A}$ receptors, may also be involved in initiating apoptosis of neurons in the AG (Meller, 2007). The 5-$HT_{2B}$ receptors may also help mediate the developmental process of programmed cell death in the AG and velum and the development of the adult central nervous system (CNS), while the faster-acting 5-$HT_3$ receptors may be involved in shutting down processes required to maintain the larval state, such as the production of NO by nitric oxide synthase (NOS) (Couper and Leise, 1996). A variety of neurotransmitters are regulators of metamorphosis and have been studied in molluscs and

include 5-HT, dopamine, and epinephrine. However, metamorphic inhibitors remained shrouded in uncertainty until the discovery of nitric oxide in the larval NS (Froggett and Leise, 1999; Hens et al., 2006; Lin and Leise, 1996).

## Nitrergic Inhibition of Metamorphosis

Multiple studies on *N. obsoletus*, the slipper limpet, *Crepidula fornicata*, and on *P. sibogae*, suggest that NO maintains the integrity of the AG throughout the larval stage in prosobranch gastropods until this neural structure is no longer needed (Lin and Leise, 1996; Thavaradhara and Leise, 2001; Gifondorwa and Leise, 2006; Bishop et al., 2008; Pechenik et al., 2007). Investigations on a variety of marine invertebrates have demonstrated that NO is a highly conserved neurotransmitter across invertebrate taxa, playing an inhibitory role in metamorphosis. Research conducted on *Phestilla sibogae*, the sea urchin *Lytechinus pictus*, the ascidian *Cnemidocarpa finmarkiensis*, the barnacle *Balanus amphirite*, and the polychaete annelid *Capitella teleta* has shown that nitrergic activity helps to maintain the larval state in multiple invertebrate phyla, but often in slightly different ways (Bishop et al., 2008; Bishop and Brandhorst, 2001; Biggers et al., 2012; Zhang et al., 2012). For example, a decrease in NOS activity induces metamorphosis in newly hatched larvae of the sea slug *Alderia willowi*, but in larvae that are two days post hatching a decrease in NOS activity instead sensitizes them to natural settlement cues rather than inducing metamorphosis directly (Romero et al., 2013).

NADPH diaphorase (NADPHd) histochemisty has revealed that the neuropil of the apical ganglion of developing *N. obsoletus* contains neuronal nitric oxide synthase (nNOS), the enzyme that makes nitric oxide from L-arginine, NADPH, and several other co-factors, leaving L-citrulline as a byproduct. Neuronal NOS is also calcium/calmodulin-dependent (Dwyer et al., 1991; Bredt and Snyder, 1994). NADPDd staining was especially strong in competent larvae but decreased after the onset of metamorphosis. NADPHd staining of the central nervous system (CNS) in juveniles showed a new pattern of staining and the absence of the AG (Lin and Leise, 1996). Later immunocytochemical work in *N. obsoletus* demonstrated 2 clusters of nNOS-immunoreactive (NOS-IR) cells in the AG and that these cells increase in number throughout larval development (Thavaradhara and Leise, 2001). All or nearly all of the 28 neurons in the AG (Dickinson and Croll, 2003) appear to express nNOS at competence (Thavaradhara and Leise, 2001), and a decrease in NOS gene expression at metamorphosis was later directly confirmed with molecular techniques (Hens et al., 2006). Metamorphosis can also be induced by inhibiting NOS or by removing NO with NO scavengers, suggesting that NO is important in inhibiting metamorphosis and maintaining the larval state and health of the AG (Froggett and Leise, 1999; Leise et al., 2001). NO is well known as an inhibitor of apoptosis in a wide variety of organisms (Gifondorwa and Leise, 2006; Peunova et al., 1996; Thavaradhara and Leise, 2001; Wang et al., 2002). More recently, the administration of various GABAergic reagents to competent larvae has suggested that GABA may also have a role to play in maintaining the larval stage in *N. obsoletus* (Biscocho and Leise, 2012). Thus, in several molluscs

and other invertebrate phyla, NO release by cells of the AG is important in maintaining the larval phenotype and regulating metamorphosis, perhaps restricting metamorphosis to favorable environments (Bishop and Brandhorst, 2001; Froggett and Leise, 1999; Pechenik et al., 2007).

## Gamma Aminobutyric Acid and Inhibition of Metamorphosis in *N. obsoletus*

Gamma aminobutyric acid (GABA) is a widespread inhibitory amino acid neurotransmitter that acts largely on $GABA_A$ receptors throughout the animal kingdom, to induce an inward chloride ion current, which then triggers a rapid inhibitory post-synaptic potential. These ligand-gated chloride ion channels can be activated by GABA agonists, such as muscimol, and can be blocked either by GABA antagonists like bicuculline or gabazine which antagonize GABA at its receptor binding site, or by the $Cl^-$ channel blocker, picrotoxin (Olsen and Sieghart, 2009). GABA is not unique to mammals; for example, Richmond et al. (1991) demonstrated that GABA was present in the major ganglia of the central nervous system (CNS) of the pond snail *Helisoma trivolvis* using immunohistochemistry and autoradiography. They also used high performance liquid chromatography (HPLC) following CNS incubation in $^3$H-glutamate to quantify the presence of GABA and to identify the ganglia in which GABA was most highly synthesized (Richmond et al., 1991).

Although GABA is widely considered to be an inhibitory neurotransmitter, it can induce settlement (on the substratum) and metamorphosis of larvae of multiple species of

abalone, as typified by *Haliotis rufescens* (Morse et al., 1979). In these instances, it appears that GABA is acting externally by mimicking the action of an algal ligand and not as an endogenous neurotransmitter. However, work done on *H. rufescens* suggested that GABA could act as an internal neurotransmitter and inhibit metamorphosis (Morse et al., 1980). To the best of our knowledge, this concept was never fully investigated in any gastropod species (Leise and Cahoon, 2012). Recently, Biscocho and Leise (2012) provided evidence that indicates that GABA can inhibit metamorphosis and maintain the larval state in *N. obsoletus*. The widespread occurrence of GABA and NO makes understanding their neuronal interactions, in the context of metamorphosis, important.

## Hypotheses Concerning the Relationship between Nitrergic and GABAergic Activity

For *N. obsoletus*, GABAergic and nitrergic activity both function to inhibit metamorphosis while serotonin induces this process (Biscocho and Leise, 2012; Leise and Cahoon, 2012). This project has attempted to uncover the specific relationship between the GABAergic and nitrergic neurons in the snail's metamorphic pathway. I hypothesize that at least three possible situations exist: 1) GABAergic neurons are upstream from nitrergic neurons, 2) nitrergic neurons are upstream from GABAergic neurons, or 3) nitrergic neurons and GABAergic neurons function in parallel pathways (Fig. 1).

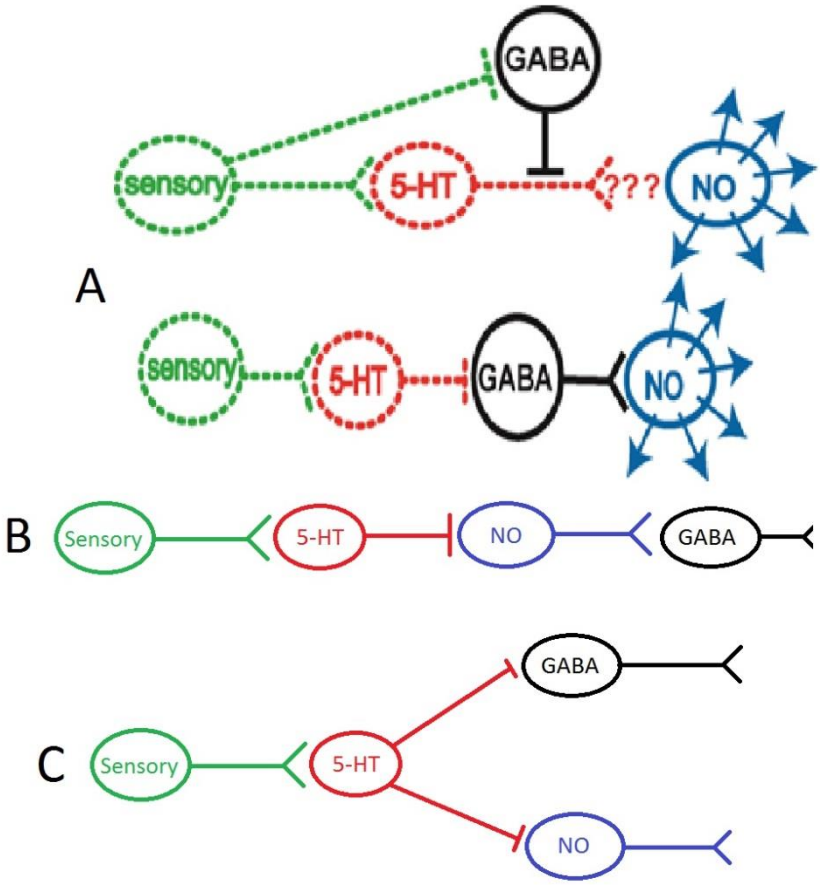


Figure. 1. Alternate Hypotheses for the Arrangement of GABAergic and Nitergic Neurons. (A) Alternate neuronal arrangements related to hypothesis 1. This pathway may be active in the larval nervous system to maintain the larval state and prevent the onset of metamorphosis. GABA may function to inhibit serotonergic neurons thereby maintaining the activity of nitergic neurons (top) or may directly stimulate nitrergic neurons to produce NO (bottom). (B) Possible neuronal arrangements related to hypothesis 2. NO may maintain the larval state by stimulating the production of GABA directly. (C) Possible neuronal arrangement related to hypothesis 3. Nitrergic and GABAergic neurons may not interact with each other directly. 5-HT may shut down NO and GABA production in parallel pathways to commit larvae to metamorphosis.

Because NO is a gaseous neurotransmitter made on demand and is not stored in vesicles, I suggest that nitrergic neurons should require more modulation or more precise physiological control and so should be downstream of GABAergic neurons (Fig. 1A). NO appears to control programmed cell death (PCD) of the apical ganglion; once a cell is committed to PCD, the process cannot be reversed (Gifondorwa and Leise, 2006; Cregan et al., 2002; Fiscus, 2002; Barres et al., 1992). Once NO is removed, programmed cell death (PCD) begins within about 6 hours (Gifondorwa and Leise, 2006, Hens et al., 2006). These findings support my hypothesis that nitrergic activity should be more tightly regulated than GABAergic activity in this system; inhibition of NOS is likely to be the final step in the series of events that triggers metamorphosis in *N. obsoletus*. I used a series of pharmacological experiments in an attempt to support hypothesis #1 and disprove #2 (Fig. 1).

Furthermore, in addition to the above experiments and in collaboration with members of the laboratory of Dr. Nadja Cech, we attempted to conduct high performance liquid chromatography-mass spectrometry (HPLC-MS) to demonstrate that GABA is present in our molluscan larvae. A better understanding of these neuronal interactions will improve our understanding of one of the least known circuits in the field of molluscan neurobiology.

# CHAPTER II

# MATERIALS AND METHODS

## Animal Acquisition and Care

During the winter, adult *N. obsoletus* were collected from the coastal mudflats at the CREST Research Center (Center for Marine Science) of UNCW in Wilmington, North Carolina and transported back to the laboratory at UNC Greensboro. In the laboratory, they were housed in glass tanks filled with artificial seawater at a salinity of approximately 31ppt, at 26$^{o}$C, and pH 8.1. One hundred animals were allocated to each tank and within a few days the animals started laying egg capsules. Animals were also stored in an environmentally controlled room at 6$^{o}$C. This kept them from reproducing and laying egg capsules until they were brought to the warmer tanks in the laboratory. Egg capsules were gathered by hand using a razor blade and a turkey baster to suck up the capsules once they were cut free of the tank wall. Egg capsules were rinsed daily in 70% ethanol, then in 0.2μm filtered Reef Crystals© artificial seawater (FIO). Hatched larvae were collected with a 75μm mesh filter and, if there were enough, were put into culture for eventual experimentation. Otherwise, larvae were backwashed into a fingerbowl that was covered in plastic wrap, and stored in the refrigerator at 7$^{o}$C. This allowed them to survive overnight to be combined with the next day's yield in a new culture.

## Larval Culture Methods

Larval cultures were created by placing a fingerbowl full of larvae adjacent to the light of a dissecting microscope and placing a black plastic cover with a square cut out of it over the fingerbowl. The light was placed against the side of the bowl lacking the black plastic and within approximately 15 minutes, healthy, swimming larvae that display positive phototaxis could be easily collected with a Pasteur pipette. Approximately 700 larvae were transferred into a 130μm mesh-bottomed plastic beaker which was then placed into a 1L glass beaker containing 600-650mL of 0.2μm filtered larval seawater (LSW), a 1:1 mixture of FIO and natural ocean water. An air pump and bubbler apparatus was attached to the beakers to keep the culture oxygenated. New cultures were fed 40mL of *Isochrysis galbana* and then subsequently, 20mL of a 1:1 mixture of *Isochrysis galbana* and *Duneliella tertiolecta* daily until they were used for an experiment (Miller and Hadfield, 1986; Gharbiah et al., 2009).

Larval cultures typically took two to three weeks to reach metamorphic competence at 26$^{o}$C. Until such time, the LSW was changed weekly. LSW in cultures also contained an antibiotic mixture of streptomycin and penicillin to reduce bacterial growth (Miller and Hadfield, 1986). Freshly changed cultures were fed 40mL of the 1:1 algal mixture. Metamorphic competence was estimated by the age of the culture (days after hatching) and by measuring the size of the animals. Cultures were usually considered to have reached metamorphic competence when the animals had an average shell length of 550μm or greater. The rate at which cultures reach competence varied

depending on the density of the culture, food availability and temperature. To measure animal size, a sample of larvae was taken from the culture and placed in deionized water in a stender dish. This caused the larvae to withdraw into their shells and become immobile. The larvae were then placed under a calibrated Olympus SZ40 dissecting microscope and the magnification increased to 40x. Fifteen to twenty animals were sized by measuring the longest length of their shells. The average size, culture age, and size range of the larvae were calculated and recorded for each experiment. In addition, each experiment contained a negative and a positive control treatment, as described below, to verify larval metamorphic competence.

**Protocol for Bath Experiments**

Animals were prepared for bath experiments after sizing by a rinse in a 10% povodine iodine solution in 90% FIO followed by four washings in FIO. Animals were then transferred to a fingerbowl from which they were aliquoted into the wells of Falcon tissue culture plates. Only animals that were healthy, actively swimming veliger larvae with intact velar lobes were selected for an experiment. Animals were left in FIO until it was replaced by an experimental or control solution; all residual FIO was removed with a Pasteur pipette before the new solutions were added to prevent dilution of experimental solutions. The time that each experimental or control solution was added to the larvae was recorded in the experimental logbook. Bath experiments were carried out in untreated 24-well plastic Falcon tissue culture plates, with 5 (Figs. 2-6, 8-11) or 10 (Fig. 7) larvae and 2 mLs of experimental or control solution allocated to each well. Each

treatment group consisted of 20-90 larvae and, if possible, experiments were repeated 3 times. The actual number of replicate wells is noted on each figure. Typical controls included FIO, 0.1mM 5-HT, 0.035mM serotonin (L5-HT), 0.25mM 7-NI and 0.2mM 7-nitroindazole (7-NI). Experiments in which control results were similar were grouped together for statistical purposes. With four exceptions (Figs. 7, 9, 10, 11), bath experiments included a positive (0.1mM 5-HT) and negative (FIO) control as well as a 0.25mM 7-NI control. In one case (Fig. 11), control groups were exposed to FIO and L5-HT for 48 hours. After 48 hours, positive controls of 0.1mM 5-HT or 0.25mM 7-NI typically yielded 80-90% and 70-80% metamorphosis respectively. When inducers, such as 7-NI, were used that were known to be weaker than 0.1mM 5-HT, the L5-HT control was included as well. Two concentrations of 7-NI were used because I found that 0.2mM 7-NI was a suboptimal metamorphic inducer. Levels of metamorphosis were higher with 0.25mM 7-NI and it was still possible to maintain the larval state with GABA or a GABA agonist.

**NOS Antagonist and GABA Agonist in Bath Experiments**

Larvae were incubated in the nitric oxide synthase (NOS) inhibitor 7-nitroindazole (7-NI) at 0.2mM for 24 hours. This exposure period and concentration were suboptimal for inducing metamorphosis, meaning that animals had not fully committed to metamorphosis and the larval state could be restored. Suboptimal metamorphosis yielded at most 40-50% metamorphosis. Following this treatment, the 7-NI solution was removed and replaced with a solution of GABA (Table 1). Animals in

the experimental group that were exposed to 7-NI and then GABA were compared to the 0.2mM 7-NI → FIO control which exhibited 35-40% metamorphosis at 48 hours. If GABAergic neurons are downstream from nitrergic neurons, I expected to see lower levels of metamorphosis in larvae transferred to GABA or a GABA agonist compared to those transferred from 7-NI to FIO.

Some experiments included exposure to 5-HT for only 5 hours, which also yielded suboptimal metamorphic induction (Figs. 5, 6, 9, 10). A 24 hour exposure to the GABA antagonist, gabazine, was also used for the same purpose.

All SNAP experiments were conducted using an additional degassed SNAP (DSNAP) control. 0.1mM SNAP was left sitting overnight to allow all the NO gas to diffuse out of the solution, leaving behind an inert byproduct. Animals exposed to DSNAP received a solution containing this inert byproduct and no NO gas.

**Table 1. GABAergic and Nitrergic Reagents Used**

| | Antagonist | Agonist/NO Donor |
|---|---|---|
| GABA | Gabazine | ---- |
| Nitric Oxide | 7-nitroindazole (NOS antagonist)<br>Aminoguanidine hemisulfate (AGH, NOS antagonist) | S-nitro, N-acetyl penicillamine (SNAP, NO donor) |

## GABA Extraction for HPLC/Mass Spectrometry

To definitively demonstrate that GABA is present in the larval nervous system of *N. obsoletus*, I conducted high performance liquid chromatography-mass spectrometry (HPLC-MS) in conjunction with Dr. Nadja Cech and her student, Mr. Scot Jarmusch. Samples were prepared by first rinsing larvae in the povodine solution as described above then transferring them to a fingerbowl with FIO. Larvae were then aliquoted into a microfuge tube on ice, with periodic removal of FIO to prevent overflow. Initially, I used a sample size of 150 larvae but we detected no GABA upon preliminary analysis. Subsequent samples were prepared using a larger sample size of 700-1000 larvae. Larvae were kept on ice until ready for extraction.

Before the addition of the extraction solvent, as much FIO was removed from the microfuge tube as possible to prevent dilution of the solvent. Larvae were ground up

with a glass pestle in 200µL absolute methanol with 0.1M HCl, an appropriate extraction solvent for GABA (Herranz et al., 1984; Dericioglu et al., 2008; Jedlicka et al., 2009; Silva et al., 2009). The microfuge tube was placed in contact with a metal grinding bowl chilled with acetone and dry ice, and then the larvae were ground into a fine slurry. Samples were then centrifuged at 10,000 rpm for 5 minutes and the supernatant transferred to another centrifuge tube that had been kept on ice. All samples were stored at -80$^{o}$C until Mr. Jarmusch analyzed them by HPLC. This project was continued by Mr. Mohamed Al-Tahir and is currently being pursued by Mr. Conner Martin and members of Dr. Cech's laboratory. Their procedures are modifications of my initial protocols and include methods for elimination of much lipid contamination. They have recently obtained encouraging results using about 240 larvae per sample. My protocol is included here because of my initial contribution to this project.

**HPLC/Mass Spectrometry Analysis**

The analysis protocol for the extraction samples was devised by Mr. Jarmusch. Each sample, dissolved in methanol, was run at a concentration of 1 mg/ml. Each sample was run through Water's Aquity Ultra-High Performance Liquid Chromatography system coupled to LTQ Orbitrap XL mass spectrometer, allowing for high resolution mass spectrometry. The method for liquid chromatography is as follows:

A- $H_2O$ (with 0.1% formic acid)

B- Acetonitrile (with 0.1% formic acid)

**Table 2. Method for Liquid Chromatography**

| Flow rate | Time | % A | % B |
|---|---|---|---|
| 0.3 mL/min | 0.0 | 25 | 75 |
| 0.3 mL/min | 3.0 | 25 | 75 |
| 0.3 mL/min | 4.0 | 100 | 0 |
| 0.3 mL/min | 4.1 | 25 | 75 |
| 0.3 mL/min | 5.0 | 25 | 75 |

## Statistical Analysis

My binomial data (larvae metamorphosed or not) are not normally distributed. To determine standard deviations (SDs) of the mean, I performed an arcsine transformation on the raw data, but means and SD were back transformed to make graphs easily understood (Sokal and Rohlf, 1995). Comparisons between treatments and controls were made by conducting Chi Square contingency table analysis on raw data. While such analysis is not as sensitive as ANOVA, it has the advantage of using raw, untransformed data. Bonferroni's method (Bonf.) was used to correct for multiple comparisons. Data were plotted with GraphPad Prism 5.0.

# CHAPTER III

# RESULTS

## Bath Experiments

Initial experiments probed ways to inhibit activity of NOS. Bath application of aminoguanidine hemisulfate (AGH), a NOS inhibitor, stimulated up to 60 percent metamorphosis in larval *C. fornicata (*Pechenik et al. 2007). However, AGH was ineffective as a metamorphic inducer for *N. obsoletus* at concentrations ranging from 0.1mM to 1.0mM, especially when compared to 5-HT or 7-NI, at both 24 and 48 hours (Fig. 2A, B). In light of these results, all nitrergic induction experiments were conducted using the neuronal NOS inhibitor 7-NI.

Several experiments were conducted to determine an optimal and then a suboptimal concentration and length of exposure for metamorphic induction by 7-NI. Timecourse experiments with bath application of 0.2mM 7-NI allowed me to deduce a suboptimal period of exposure for larval *N. obsoletus*. Percent metamorphosis after 2, 4, 6, and 8 hours of exposure to 0.2mM 7-NI were insignificant, so all future experiments with 7-NI were conducted with only a 10 or 24 hour exposure (Fig 3A, B). My experiments also demonstrated that bath applications of 0.25mM 7-NI triggered metamorphosis most strongly; concentrations higher than 0.25mM appeared to be toxic to

competent larvae of *N. obsoletus* (Fig. 4). Experiments using a 24 hour exposure to 0.2mM 7-NI to suboptimally induce metamorphosis followed by application of 0.5mM GABA allowed me to investigate the relationship between GABA and NO in the metamorphic circuit of *N. obsoletus* (Figs. 5, 6). As expected, induction with a 5 hour exposure to 0.1mM 5-HT followed by exposure to 0.5mM GABA (Figs. 5, 6) demonstrated reduced levels of metamorphosis compared to a transition to FIO. Induction with 0.2mM 7-NI followed by exposure to GABA showed reduced levels of metamorphosis in one experiment compared to L5-HT controls (Fig. 5), but not in others (Fig. 6).

To determine if a NO donor could restore the larval state following the triggering of metamorphosis with a GABAergic reagent, it was necessary to determine the concentration of Gabazine (GBZ), a GABA antagonist, that was effective at triggering high percentages of metamorphosis. A 48 hour exposure to multiple concentrations of Gabazine was used to do this. Results showed that 1mM Gabazine was most effective at triggering metamorphosis while all other concentrations had little effect (Fig. 7A, B).

Experiments were conducted with GBZ and 7-NI to investigate a possible synergistic interaction between these two reagents. To accomplish this, larvae were exposed to 0.2mM 7-NI alone, 1mM GBZ alone, and a combination of these reagents (Fig. 8A, B). In results from the two experiments conducted, larvae exposed to this combination of reagents displayed a higher percentage of metamorphosis at both 24 and 48 hours (Fig. 8) than larvae exposed to either 7-NI or GBZ individually.

To demonstrate that nitrergic neurons are downstream from GABAergic ones, I exposed larvae to the GABA antagonist GBZ in 3 experiments (Figs. 9-11) to induce metamorphosis and then subsequently treated larvae with the NO-donor SNAP to inhibit metamorphosis and thus maintain the larval state. Controls included degassed SNAP (DSNAP) which should have had no effect on larvae (Froggett and Leise, 1999). GBZ showed no effect on metamorphosis in one experiment (Fig. 9), nor did SNAP. In a second experiment, SNAP again failed to inhibit metamorphosis (Fig. 10), although SNAP appeared to act as expected in a final experiment (Fig. 11). However, in this experiment (Fig. 11) statistical analysis again determined that SNAP had no effect on GBZ induction of metamorphosis.

**HPLC/Mass Spectrometry**

HPLC-MS analysis of several samples of larvae has failed to detect any GABA thus far. However, efforts are underway to refine the extraction technique to improve detection.

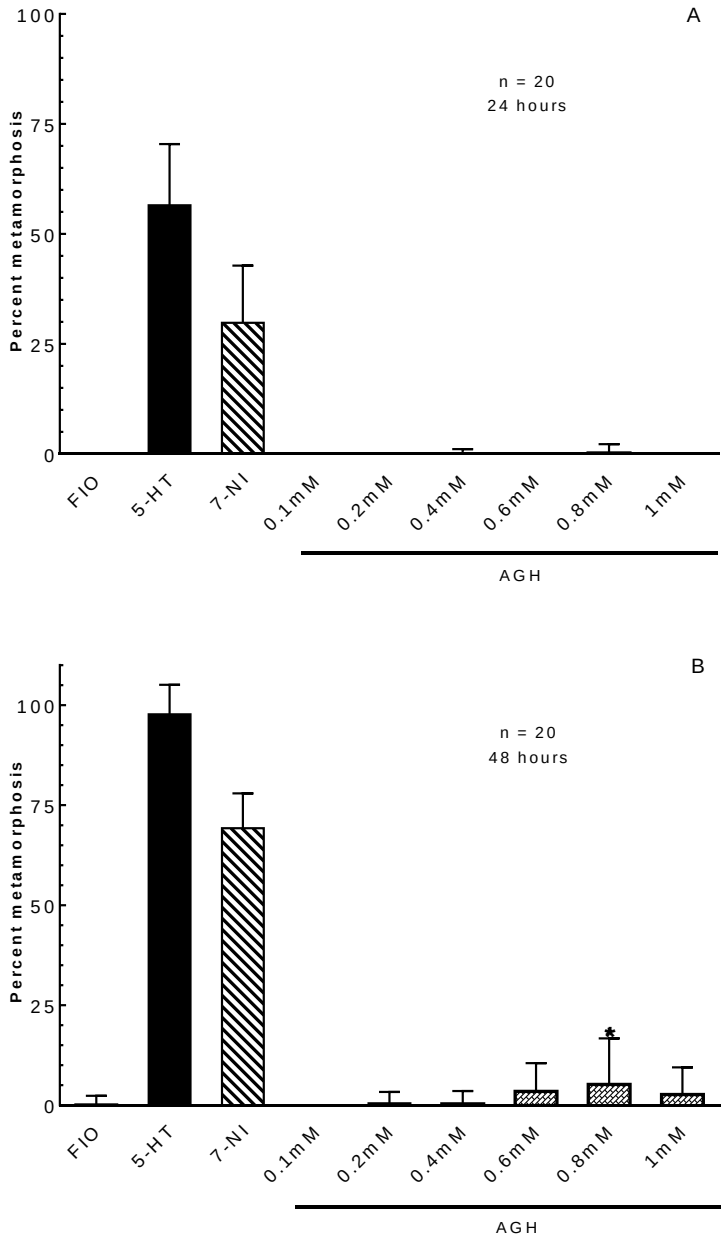


Figure 2. Bath Application of AGH did not Yield Useful Levels of Metamorphosis. Competent larvae were exposed for a total of 48 hours to AGH and while one treatment (*) yielded a level of metamorphosis at 48 hours (B) that was significantly different from FIO, this was too low to be useful in future experiments ($X^2 = 8.73$, $\alpha = 0.008$, with Bonferroni's correction, for 0.8mM). n= number of wells per group.

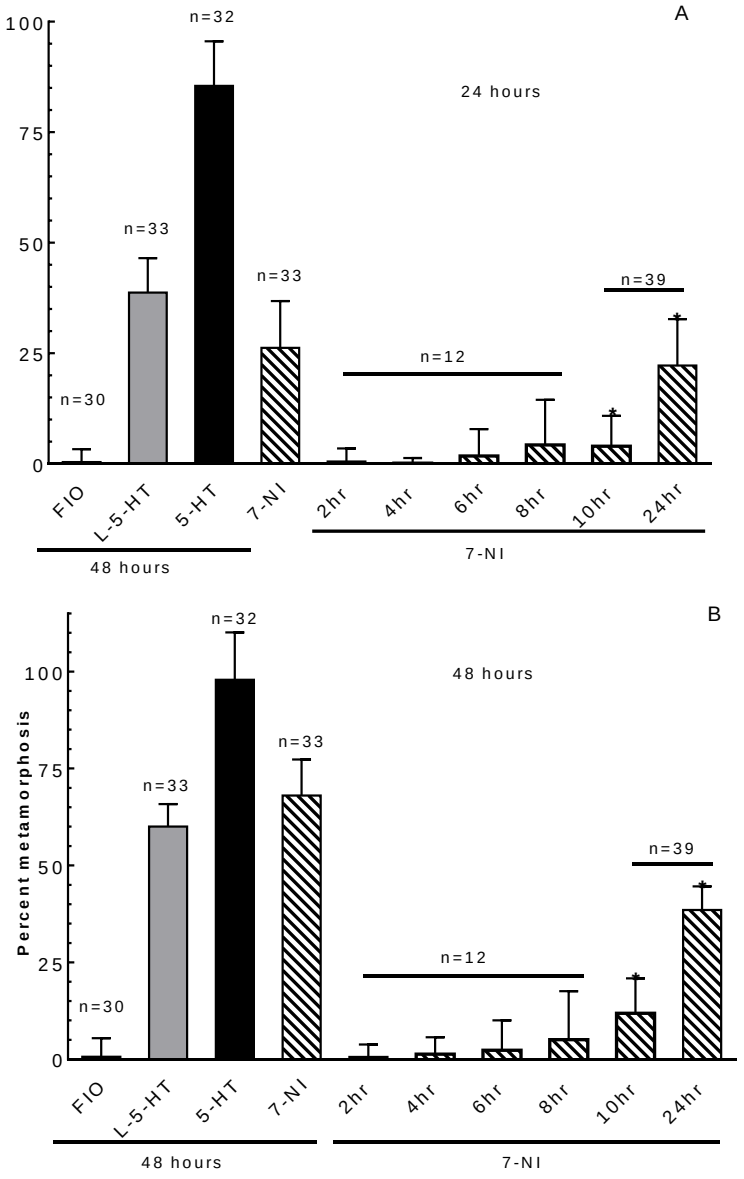


Figure 3. Timecourse to Determine an Effective and a Sub-optimal Exposure Time for Eliciting Metamorphosis with 0.2mM 7-NI. Percent metamorphosis was too low at 2-8 hours of exposure to be useful in future experiments. Animals exposed to 7-NI for 24 hours showed a significantly higher percentage of metamorphosis than animals exposed for 10 hours at both 24 (A) and 48 (B) hours ($X^2$=20.5 at 24 hours, 22.5 at 48 hours). 10 hours of exposure to 7-NI at 48 hours showed a significant difference compared to the FIO control ($X^2$= 16.9, Bonf. $\alpha$ =0.008). Percent metamorphosis was scored at 24 (A) and 48 (B) hours; n= number of wells per group. Bars with * have means that are significantly different from FIO.

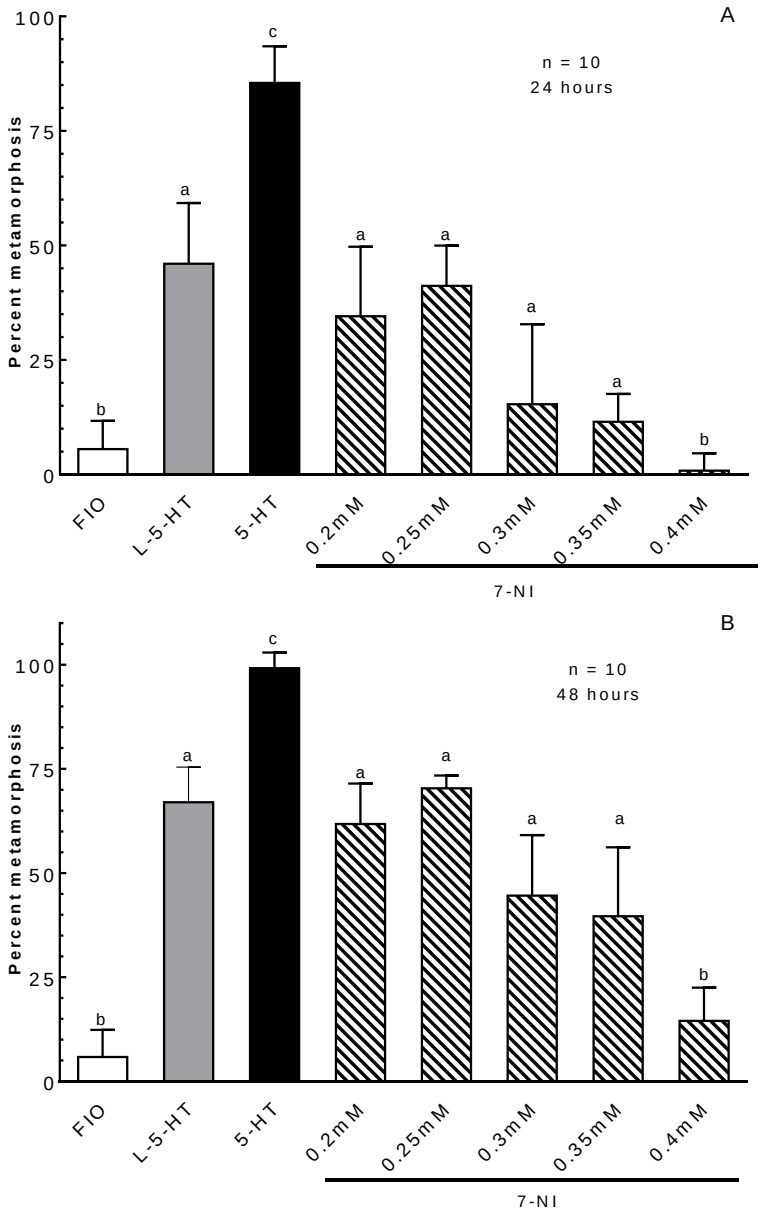


Figure 4. A Determination of the Concentration of 7-NI that Most Strongly Triggers Metamorphosis. 0.2mM, 0.25mM, 0.3mM, and 0.35mM 7-NI induced levels of metamorphosis similar to those elicited by the L5-HT control group at 24 (A) and 48 (B) hours, but the means for these four treatments of 7-NI were not significantly different when compared to each other ($X^2$=0.84, Bonf. $\alpha$=0.01). n= number of wells per group. Bars with different letters have significantly different means as determined by the Chi Square test at $p < 0.01$ or better; bars with the same letter have means that are not statistically different.

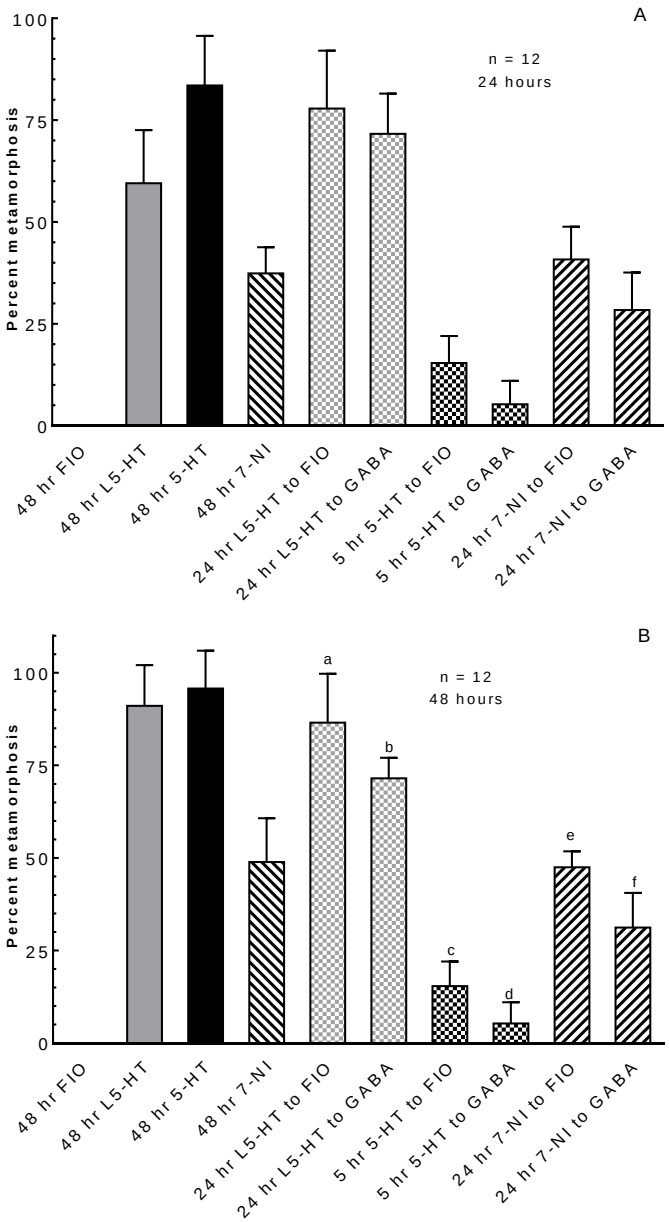


Figure 5. Inhibition of Metamorphosis by GABA in Larvae Exposed to L5-HT, 5-HT, and 7-NI. Suboptimal induction using a 5 hour exposure to 5-HT followed by 0.5mM GABA (5HT to GABA) significantly decreased metamorphosis at 48 hours (B) compared to the 5-HT to FIO control ($X^2$=2.35, $\alpha$=0.05). Similar results were observed for L5-HT to GABA (compared to L5-HT to FIO) and 7-NI to 0.5mM GABA (compared to 7-NI to FIO) ($X^2$=1.21, 1.71 respectively). For treatments labeled FIO, L5-HT, 5-HT, and 7-NI larvae remained in the original solution for 48 hours. For 24 hour L5-HT and 7-NI, solutions were changed to either FIO or GABA. n= number of wells per group. On the 48 hour graph, bars with different letters have significantly different means as determined by the Chi Square test at $p < 0.05$ or better.

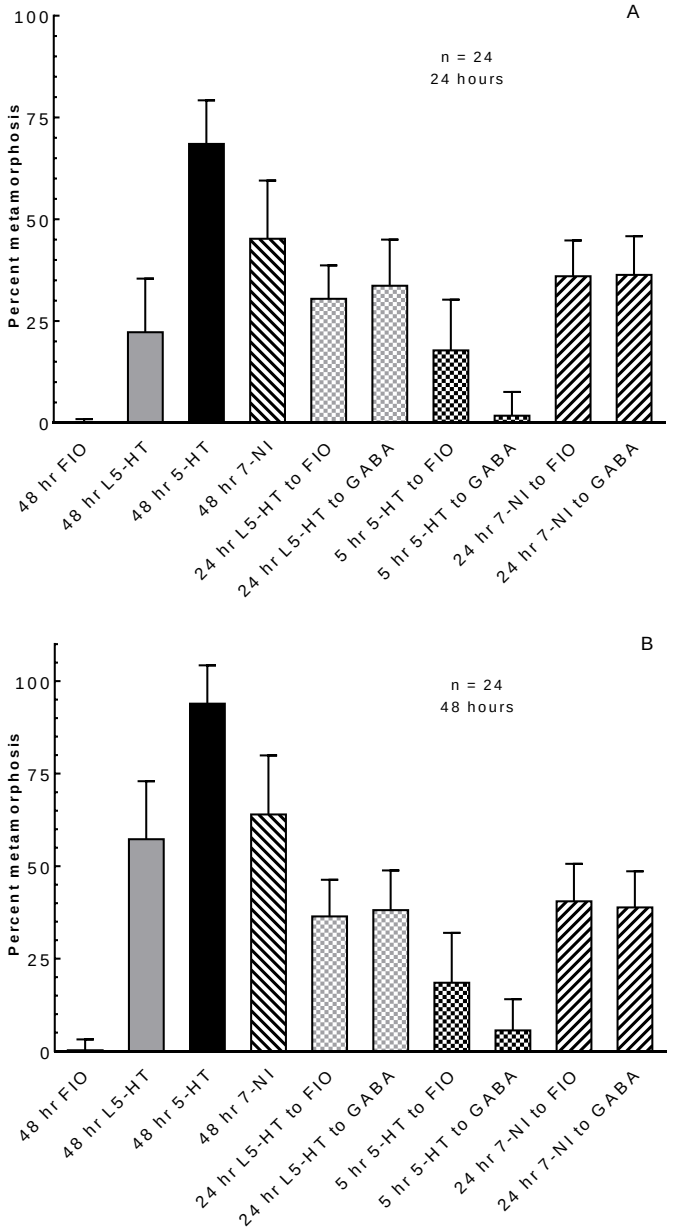


Figure 6. GABA Failed to Inhibit Metamorphosis in Larvae Exposed to 7-NI. An attempt to repeat the experiments shown in Fig. 5, to determine if inhibition of metamorphosis by GABA is effective after NOS inhibition (induction of metamorphosis using 7-NI). Larvae were exposed to either L5-HT or 7-NI for 24 hours and then solutions were replaced with FIO or 0.5mM GABA. Larvae were exposed to 5-HT for 5 hours, as indicated. Control groups (FIO, L5-HT, 5-HT) remained in the original solution for 48 hours. Metamorphosis was scored at 24 (A) and 48 (B) hours; n= number of wells per group. Unlike the experiment shown in Fig. 5, GABA was only effective at reducing serotonergically induced metamorphosis when compared to 5-HT to FIO (5-HT to FIO vs. 5-HT to GABA, $X^2$=6.91; 7-NI to FIO vs. 7-NI to GABA, $X^2$=0.003; $\alpha$=0.05).

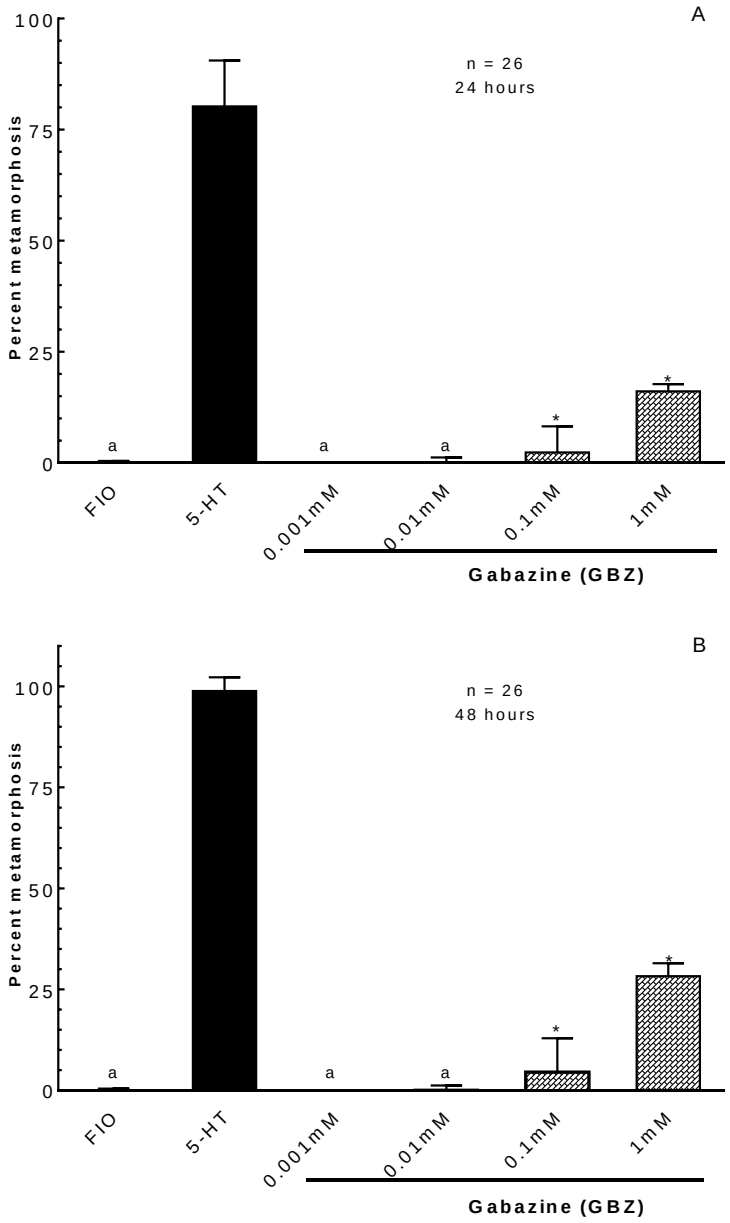


Figure 7. Determination of Effective GABAzine Concentration for Triggering Metamorphosis. n= number of wells per group. Concentrations less than 0.1mM did not trigger significant levels of metamorphosis when compared to FIO ($X^2$=27.5, Bonf. α =0.125); 1mM induced significantly more metamorphosis compared to 0.1mM ($X^2$=26.9, α=0.05 at 48 hours). Larvae were exposed to all reagents for 48 hours. Bars with different letters have significantly different means as determined by the Chi Square test at $p < 0.05$ or better. Bars with * have means that are significantly different from FIO.

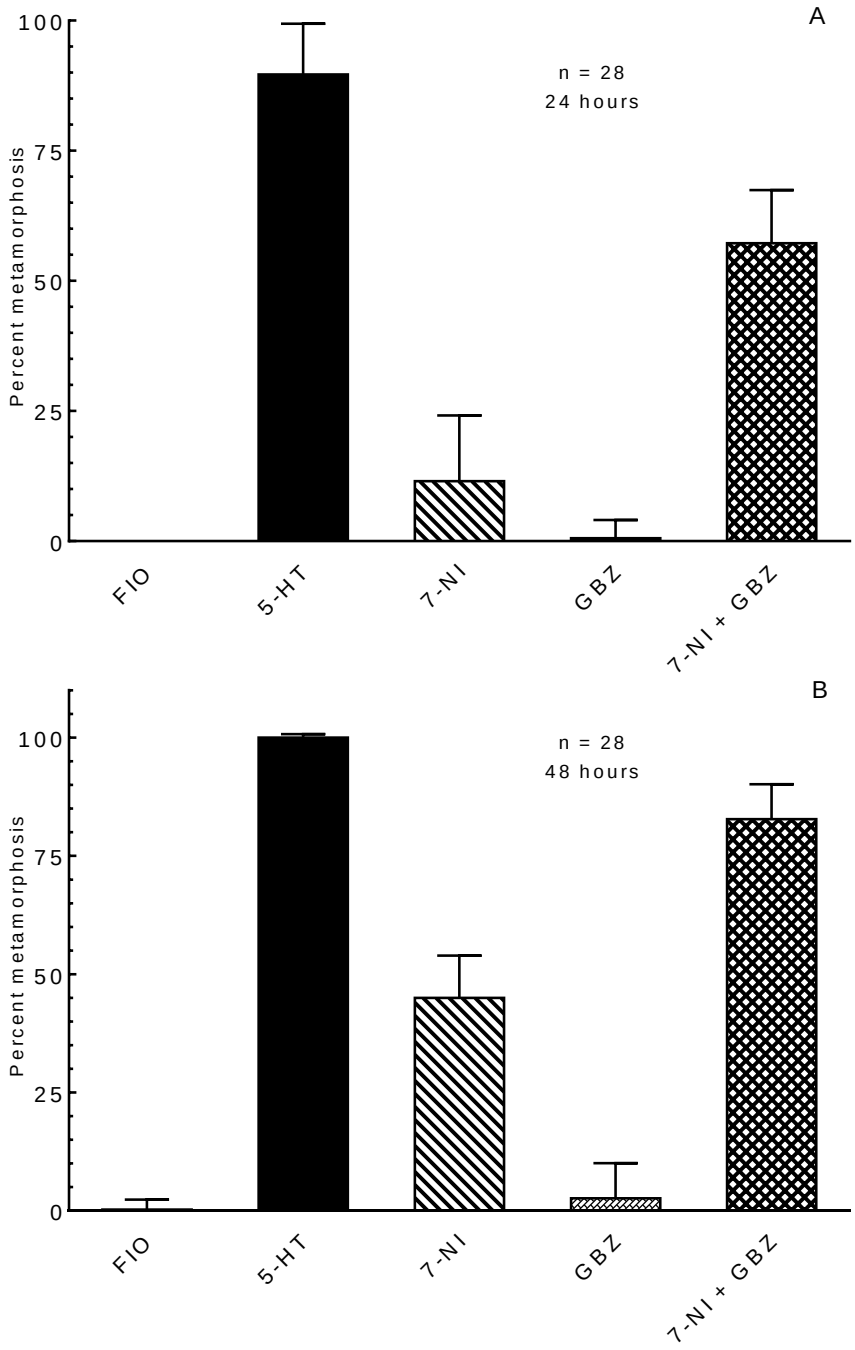


Figure 8. 7-NI and GABAzine have a Synergistic Effect on the Onset of Metamorphosis. A combination of these two reagents induces more metamorphosis than either does alone. Larvae were exposed to all reagents for 48 hours; n= number of wells per group.

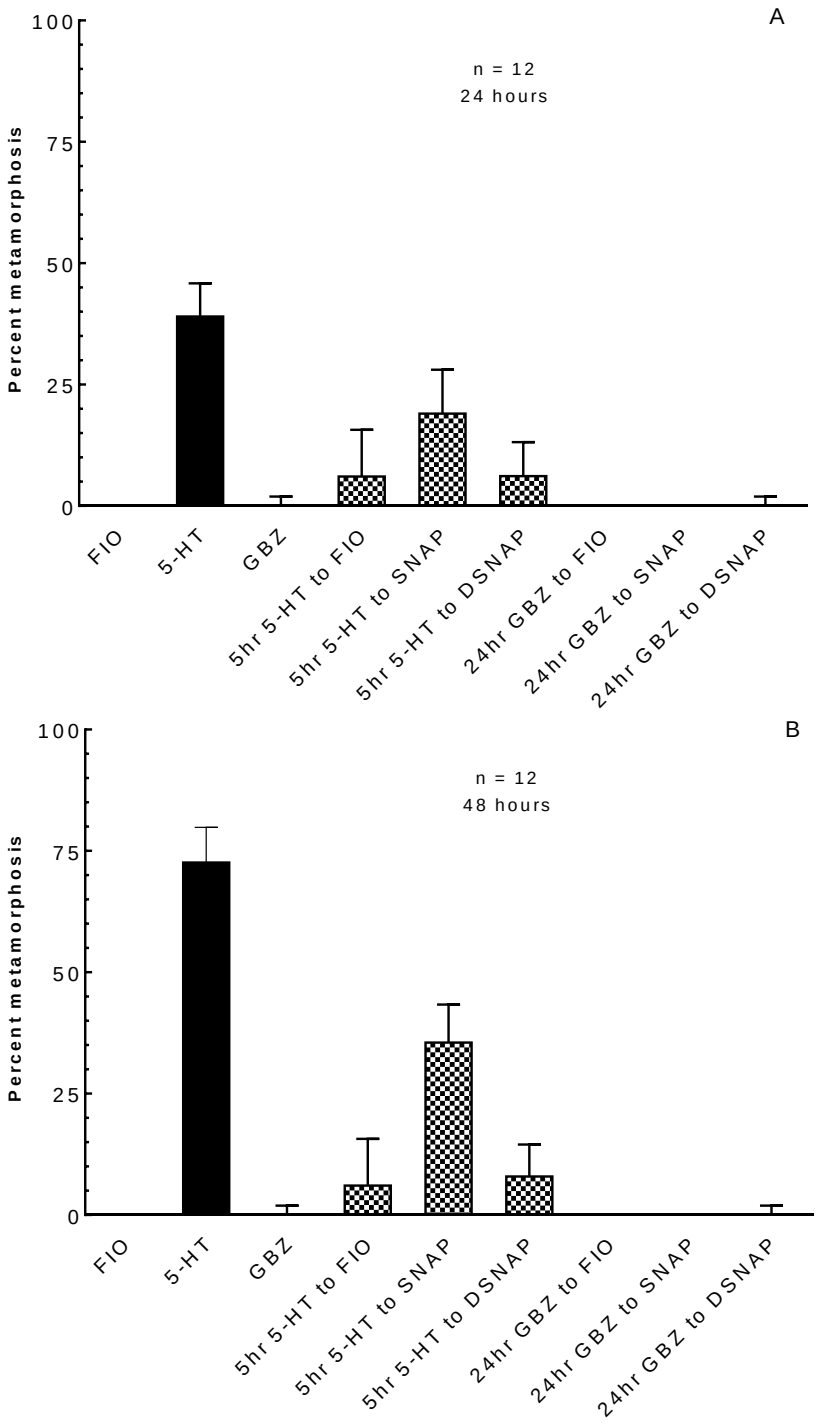


Figure 9. SNAP Failed to Inhibit Metamorphosis Triggered by GABAzine. At both 24 (A) and 48 (B) hours, GBZ induced no significant levels of metamorphosis relative to the FIO control in any application (48 hour or 24 hour exposure). n= number of wells per group.

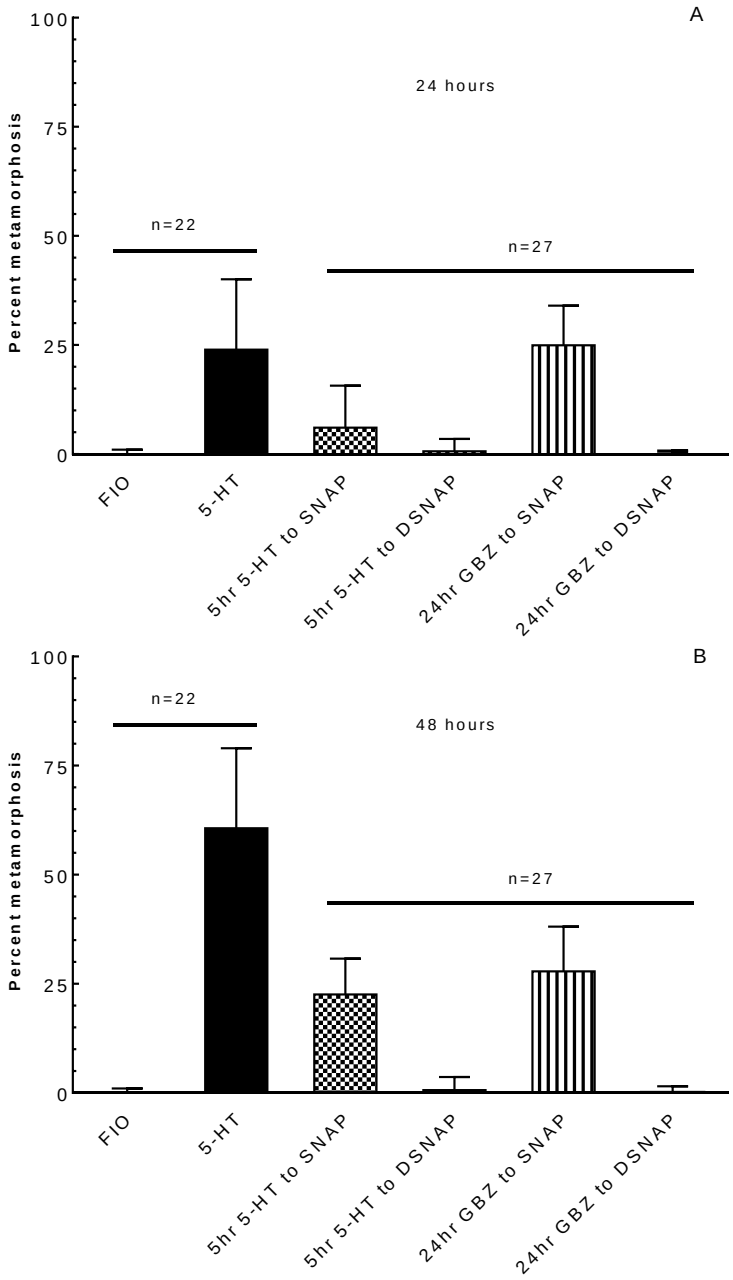


Figure 10. SNAP Failed to Inhibit Metamorphosis Triggered by GABAzine. At 48 (B) hours, SNAP failed to inhibit metamorphosis. Larvae in FIO and 5-HT controls were exposed to those reagents for 48 hours. Solutions of SNAP were changed every 6 hours to ensure a relatively stable concentration of NO. Animals in DSNAP remained in this solution for the remainder of the experiment. n= number of wells per group.

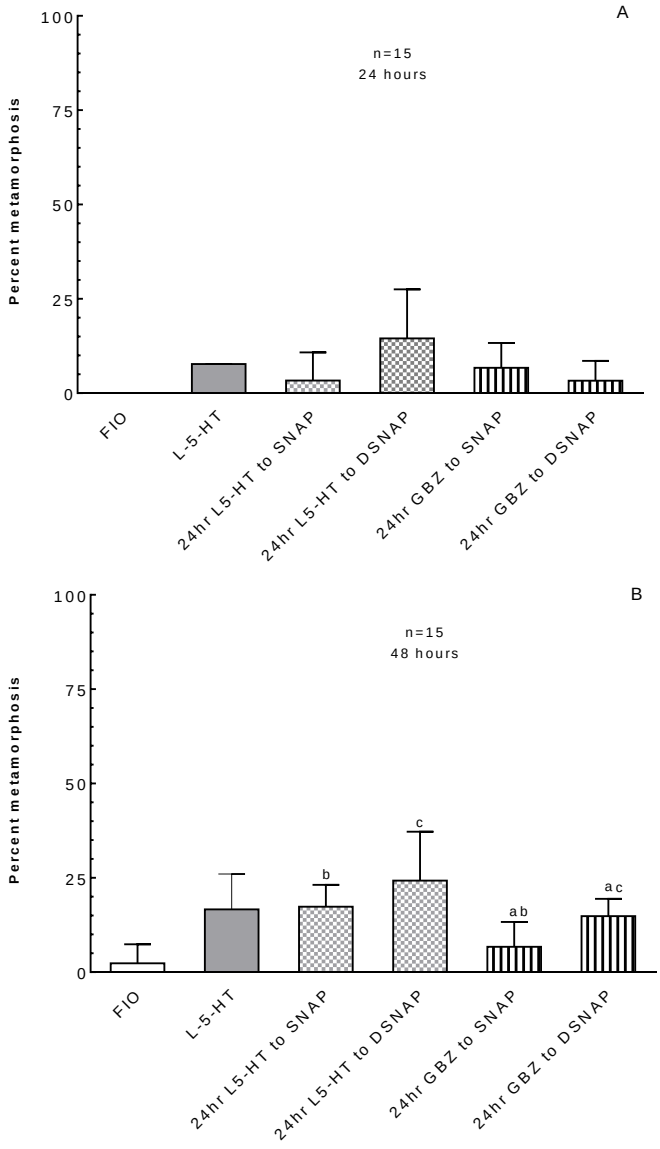


Figure 11. SNAP Inhibited Metamorphosis Triggered by GABAzine. At 48 hours (B) SNAP had not significantly reduced metamorphosis in larvae exposed to GBZ for 24 hours (GBZ to SNAP) compared to DSNAP control (GBZ to DSNAP) ($X^2$=1.44, α=0.05). Percent metamorphosis for both experimental groups was similar to the percent metamorphosis observed in L5-HT controls (L5-HT to SNAP compared to GBZ to SNAP: $X^2$=2.69, α=0.05; L5-HT to DSNAP compared to GBZ to DSNAP: $X^2$=3.42, α=0.05). SNAP was replaced every 6 hours to maintain a constant NO concentration. n= number of wells per group. Bars with different letters have significantly different means as determined by the Chi Square test at $p < 0.05$ or better.

# CHAPTER IV

# DISCUSSION

For *N. obsoletus,* GABAergic and nitrergic activity both function to inhibit metamorphosis while serotonin induces metamorphosis (Biscocho and Leise, 2012; Leise and Cahoon, 2012). Both GABA and NO are involved in maintaining the larval state in *N. obsoletus* and inhibition of the activity of either neurotransmitter will trigger metamorphosis which results in the destruction of the AG (Fig. 5, Froggett and Leise 1999, Gifondorwa and Leise 2006, Biscocho 2013). However there is still little understanding regarding the relationship between these two neurotransmitters in the CNS of *N. obsoletus*. Three possible relationships exist: 1) GABAergic neurons are upstream from nitrergic neurons, 2) nitrergic neurons are upstream from GABAergic neurons, or 3) nitrergic neurons and GABAergic neurons function in parallel pathways (Fig. 1).

In my experiments, larvae were scored as metamorphosed once they had lost the velar lobes. A better analysis would have been a morphological investigation of the AG. In animals that carried metamorphosis through to completion, the AG would be absent. I was not able to do this, but an analysis of the CNS of the larvae used in experiments with 7-NI activation/GABA inhibition of metamorphosis and in experiments with GBZ activation/SNAP inhibition of metamorphosis, should show that the AG is still intact because any metamorphosis triggered by 7-NI or GBZ would be stopped by GABA or

NO. The presence or absence of the AG would serve as better evidence to determine whether metamorphosis had been halted in these experiments.

In bath experiments, larvae were exposed to different types of metamorphic triggers, a GABA antagonist (GBZ) or a NOS inhibitor (7-NI), for 24 hours to induce low levels of metamorphosis. After 24 hours, the inductive reagent was replaced with GABA or the NO donor SNAP. Whether or not metamorphosis is halted by this second step should indicate a probable layout of the metamorphic pathway. If a GABA antagonist followed by a NO donor elicited metamorphic levels that are lower than those obtained with the control (exposure to only the inducer) then it is probable that NO-containing neurons are downstream from GABAergic neurons. Conversely, if a NOS inhibitor was replaced by GABA and levels of metamorphosis were lower than those of the controls, then it is more probable that the GABAergic neurons are downstream from nitrergic neurons in the metamorphic pathway.

More simply put, treatment with a GABA antagonist followed by a successful maintenance of the larval state with a NO donor would demonstrate that the nitrergic neurons are farther downstream than the GABAergic neurons in the larval metamorphic circuit. Conversely, treatment with a NOS inhibitor to induce metamorphosis followed by a successful GABA maintenance of the larval state would strongly suggest that the GABAergic neurons are downstream from the nitrergic neurons in this pathway.

However, the 3 experiments shown in Figs. 9-11 were inconclusive. GBZ appeared to have no effect on larvae in two experiments (Figs. 9, 10), possibly because

these animals were too young. Given the relatively low level of metamorphosis seen at 48 hours in response to 0.1mM 5-HT (<75%) these larvae may have had incompletely developed neural circuits. Results of my final experiment (Fig. 11) are encouraging, suggesting that with further repetition with competent larvae, one might see significant retention of the larval state with SNAP after a GBZ induction, but this experiment also had insignificant results after statistical analysis.

Results of an experiment using both GBZ and 7-NI in tandem to induce metamorphosis supports earlier work suggesting that GABA acts as an inhibitory neurotransmitter in larval *N. obsoletus* (Biscocho, 2013 and Fig. 8). However, all of my results would be bolstered by an independent verification of the presence of GABA in these larvae. HPLC-MS was one way to do this and should have allowed us to demonstrate that GABA is indeed present in the larval nervous system. If we had been able to show that GABA was present, and perhaps quantify the amount present per animal, then we could say with more confidence that GABA is involved in the larval NS, modulating metamorphosis.

Unfortunately, samples run through HPLC-MS did not detect GABA. Because larvae are ground up whole (the whole body and shell) it is possible that elements of the viscera or other dissolved organics were masking the GABA signal (Mr. Mohamed Al-Tahir, pers. comm.). Separation of larval components into aqueous and organic (largely non-polar molecules) phases has recently allowed Dr. Cech's students to obtain our first positive result. Ongoing work continues to optimize the signal-to-noise ratio. It is also

possible that enzymes released by the grinding are converting GABA to glutamate. Thus, my procedure has been modified such that grinding occurs in the acetone/dry ice mixture and is kept cool over the course of the grinding period; we have also attempted more rapid grinding for only a minute or less with the goal of limiting the metabolism of GABA in the sample. Various alternate protocols, such as altering the acid concentration, changing the initial extraction solvent or removing and grinding up only the head and nervous system are also being used to try to improve the quality of the current results.

Original experiments performed by Leise and colleagues demonstrated that most typical NOS inhibitors were effective only when injected directly into larvae (Froggett and Leise, 1999). Subsequent work with more modern NOS inhibitors, such as 7-NI, demonstrated effectiveness in bath application (Leise et al., 2004). Other investigators have used AGH with mixed results (Pechenik et al, 2007), but my experiments clearly demonstrated that unlike 7-NI, AGH was ineffective at triggering metamorphosis in *N. obsoletus*.

Comparisons of preliminary data with results from earlier experiments demonstrated that 0.2mM 7-NI did not trigger the same levels of metamorphosis as previously determined, so I conducted experiments to identify concentrations at which 7-NI induced metamorphosis strongly. In experiments to determine if SNAP would inhibit GABAergic initiation of metamorphosis (Fig 9), the origin of the GBZ appeared to make a significant difference, with GBZ obtained from Abcam failing to trigger metamorphosis, while GBZ obtained from Sigma-Aldrich, Inc. being more

physiologically useful. The age of the larvae may have also been an issue, meaning that they were not old enough to be competent to metamorphose (Figs. 8, 10), or that sensitivity to GBZ may change over time. In some experiments, larvae did not appear healthy in 1mM gabazine; it may have been toxic, which may have had an effect on metamorphic induction as well. In Fig. 10, inhibition of metamorphosis by SNAP was not observed until 48 hours because SNAP needs approximately 24 hours before it takes effect and because larvae were in GBZ for the first 24 hours.

The preponderance of pharmacological data I collected does not support my hypothesis that NO is downstream from GABA in the metamorphic pathway in *N.* obsoletus. While GABA did significantly reduce metamorphosis in larvae exposed to 7-NI (Fig. 5B), I was unable to replicate these results (Fig. 6) and SNAP did not significantly reduce metamorphosis compared to L5-HT and degassed SNAP (DSNAP) controls (Fig. 11). That a combination of 7-NI and GBZ triggered higher percentages of metamorphosis than either reagent does alone provides some evidence that NO and GABA play a linked role in regulating the onset of metamorphosis in the larvae of *Nassarius* (Fig. 8). These data also raise interesting questions regarding the importance of GABA and NO in the metamorphic pathway. 7-NI triggered higher levels of metamorphosis than did GBZ alone. This may indicate that NO is the deciding factor in the decision to go through metamorphosis. Additionally, since the response to GBZ is so weak, it is possible that GABAergic neurons do not directly innervate nitrergic neurons; other neurotransmitters or neurohormones may play intermediary roles to link the actions

of GABA and NO. Given the data, hypothesis #3, that NO and GABA function in parallel pathways to influence the onset of metamorphosis, is most likely.

The data produced by these experiments does warrant further investigation. There were some issues with the larvae and reagents that may have affected the outcome of some experiments. GBZ was difficult to get into solution, requiring heating of the solution and the use of an agitator for several minutes to fully dissolve the reagent. It is possible that in earlier experiments with GBZ that not all of it went into solution and so the final concentration may have been lower than intended. In experiments graphed in Figs. 9-11, the percent metamorphosis observed in the 5-HT groups was much lower than normal (compare to Figs. 2-7), raising the question of the competency of the larvae to metamorphose. Precompetent larvae would not respond as strongly to metamorphic triggers, if they respond at all, and so the full effect of 5-HT and GBZ may not have been exerted on the animals. Additional repetition of the later experiments (Figs. 9-11) using older larvae in larger numbers may clarify the results of this study.

Recent work has led us to suspect a major evolutionary divergence in the use of GABA and its receptors between Atlantic and Pacific species of gastropod molluscs, particularly between those living in rocky intertidal and subtidal areas versus those that live on mudflats (Leise and Cahoon 2012; Morse et al. 1980). On the Pacific coast, which is largely rocky intertidally and subtidally and where *N. obsoletus* is an invasive species and not widespread, in species such as *H. rufescens*, GABA acts externally to induce metamorphosis. However, research has shown that after prolonged exposure to

GABA and at concentrations above 0.001mM, GABA inhibits developmental metamorphosis. It is possible that GABA is acting as an internal inhibitor under these conditions similar to the proposed action of GABA in *N. obsoletus* (Morse et al. 1980). Thus, even in Pacific coast species, GABA could have dual roles, though this has not yet been investigated.

# REFERENCES


Barres, B. A., Hart, I. K., Coles, H. S. R., Burne, J. F., Voyvodic, J. T., Richardson, W. D., & Raff, M. C. (1992). "Cell Death and Control of Cell Survival in the Oligodendrocyte Lineage". Cell **70**, 31–46.

Biggers, W. J., Pires, A., Pechenik, J. A., Johns, E., Patel, P., Polson, T., & Polson, J. (2012). "Inhibitors of nitric oxide synthase induce larval settlement and metamorphosis of the polychaete annelid *Capitella teleta*". Invertebrate Reproduction and Development **56**(1), 1–13.

Biscocho, Dhani, M.S. GABA is an Inhibitory Neurotransmitter in the Neural Circuit Regulating Metamorphosis in *Ilyanassa obsoleta.* (2013) Directed by Dr. Esther Leise. 52pp

Biscocho, D., E. M. Leise, Carter, C.S. and Hwang, H. A. (2012). "Evidence for GABA as an Inhibitory Neurotransmitter in the Neural Circuit Regulating Metamorphosis in the Marine Mollusc *Ilyanassa obsoleta"*. 10th International Congress of Neuroethology, College Park, MD, Frontiersin.org.

Bishop, C. D., and B. P. Brandhorst (2001). " NO/cGMP Signaling and HSP90 Activity Represses Metamorphosis in the Sea Urchin *Lytechinus pictus*." Biological Bulletin **201**: 394-404.

Bishop, C. D., Pires, A., Norby, S.-W., Boudko, D., Moroz, L. L., & Hadfield, M. G. (2008). "Analysis of nitric oxide guanosine monophosphate signaling during metamorphosis of the nudibranch *Phestilla sibogae* Bergh (Gastropoda: Opisthobranchia)". Evolution and Development **10**(3), 288–299.

Bonar, D. B., & Hadfield, M. G. (1974). "Metamorphosis of the Marine Gastropod *Phestilla Sibogae* Berg (Nudibranchia: Aeolidacea). I. Light and Electron Microscopic Analysis of Larval and Metamorphic Stages". Journal of Experimental Marine Biology and Ecology **16**, 227–255.

Bouchet, P. and Rocroi, J. P. (2005). "Classification and Nomenclator of Gastropod Families". Malacologia 47(1-2), 1-397.

Bredt, D. S., & Snyder, S. H. (1994). "Nitric Oxide: A Physiologic Messenger Molecule". Annual Review of Biochemistry 63, 175–195.

Chia, F.-S. and Koss, R. (1988). "Induction of settlement and metamorphosis of the veliger larvae of the nudibranch, *Onchidoris bilamellata.* International Journal of Invertebrate Reproduction and Development 14, 53-70.

Collier, J. (2002). Abibliography of the marine mud snail *Ilyanassa obsoleta.* Invertebrate Reproduction and Development 42(2-3), 95-110.

Connor, M. S., J. M. Teal and I. Valiela (1982). "The effect of feeding by mud snails, *Ilyanassa obsoleta* (Say), on the structure and metabolism of a laboratory benthic algal community." Journal of Experimental Marine Biology and Ecology **65**(1): 29-45.

Couper, J. M. and E. M. Leise (1996). "Serotonin injections induce metamorphosis in larvae of the gastropod mollusc *Ilyanassa obsoleta*." Biological Bulletin **191**: 178-186.

Cregan, S. P., A. Fortin, J. G. MacLaurin, S. M. Callaghan, F. Cecconi, S. W. Yu, T. M. Dawson, V. L. Dawson, D. S. Park, G. Kroemer, and R. S. Slack. 2002. "Apoptosis-inducing factor is involved in the regulation of caspase-independent neuronal cell death". J. Cell Biol. **158**:507-517.

Dawson, T.M., Bredt, D.S. Fotuhi, M., Hwang, P.M. and Snyder, S.H. (1991). "Nitric oxide synthase and neuronal NADPH diaphorase are identical in brain and peripheral tissue". Proceeding of the National Academy of Science **88**, 7797-7801.

Dericioglu, N., C. L. Garganta, O. A. Petroff, D. Mendelsohn and A. Williamson (2008). "Blockade of GABA synthesis only affects neural excitability under activated conditions in rat hippocampal slices." Neurochemistry International **53**(1-2): 22-32.

Dickinson, A.J.G. and Croll, R.P. (2003). "Development of the Larval Nervous System of the Gastropod *Ilyanassa obsoleta"*. J. comp. Neurol. **466**, 197-218.

Dwyer, M. A., Bredt, D. S., & Snyder, S. H. (1991). "Nitric Oxide Synthase: Irreversible Inhibition by L-NG-Nitroarginine in Brain in Vitro and in Vivo". Biochemical and Biophysical Research Communications **176**(3), 1136–1141.

Fiscus, R. R. (2002). "Involvement of cyclic GMP and protein kinase G in the regulation of apoptosis and survival in neural cells". Neurosignals **11**(4), 175–190.

Froggett, Stephan J. and Esther M. Leise (1999). "Metamorphosis in the Marine Snail *Ilyanassa obsoleta*, Yes or NO?". Biological Bulletin **196**: 57-62.

Gharbiah, M., Cooley, J., Leise, E. M., Nakamoto, A., Rabinowitz, J. S., Lambert, J. D., & Nagy, L. M. (2009). "Induction of Larval Metamorphosis in the Snail *Ilyanassa*". Cold Spring Harbor Protocols **1**.

Gifondorwa, D. J. and E. M. Leise (2006). "Programmed cell death in the apical ganglion during larval metamorphosis of the marine mollusc *Ilyanassa obsoleta*." Biological Bulletin **210**(1): 109-120.

Hadfield, M.G and Koehl, M.A.R. (2004). "Rapid behavioral responses of an invertebrate larva to dissolved settlement cue". Biol. Bull. **207**(1), 28-43.

Hadfield, M. G., E. A. Meleshkevitch and D. Y. Boudko (2000). "The apical sensory organ of a gastropod veliger is a receptor for settlement cues." Biological Bulletin **198**: 67-76.

Hens, M. D., K. A. Fowler and E. M. Leise (2006). "Induction of metamorphosis decreases nitric oxide synthase gene expression in larvae of the marine mollusc *Ilyanassa obsoleta* (Say)." Biol Bull **211**(3): 208-211.

Herranz, A. S., J. Lerma and R. M. Delrio (1984). "Determination of Gamma-Aminobutyric Acid in Physiological Samples by a Simple, Rapid High-Performance Liquid-Chromatographic Method." Journal of Chromatography **309**(1): 139-144.

Jedlicka, S. S., M. Dadarlat, T. Hassell, Y. Z. Lin, A. Young, M. Zhang, P. Irazoqui and J. L. Rickus (2009). "Calibration of Neurotransmitter Release from Neural Cells for Therapeutic Implants." International Journal of Neural Systems **19**(3): 197-212.

Kelaher, Brendan P., Levinton, Jeffrey S., Hoch, Matthew J. (2003). "Foraging by the mud snail, *Ilynasassa obsoleta* (Say), modulates spatial variation in benthic community structure." Journal of Experimental Marine Biology and Ecology 292, 139-157.

Kempf, S. C., L. R. Page and A. Pires (1997). "Development of serotonin-like immunoreactivity in the embryos and larvae of nudibranch mollusks with emphasis on the structure and possible function of the apical sensory organ." Journal of comparative Neurology **386**: 507-528.

Leise, E. M. and L. B. Cahoon (2012). "Neurotransmitters, Benthic Diatoms and Metamorphosis in a Marine Snail". Snails: Biology, Ecology and Conservation, Nova Science Publishers, Inc.**:** 1-43.

Leise, E. M., S. J. Froggett, J. Nearhoof and L. B. Cahoon (2009). "Diatom cultures induce and inhibit larval metamorphosis in the marine gastropod *Ilyanassa obsoleta* (Say)." Journal of Experimental Marine Biology and Ecology **379**: 51-59.

Leise, E.M., Kempf, S.C., Durham, N.R. and Gifondorwa, D.J. (2004). "Induction of metamorphosis in the marine gastropod *Ilynassa obsoleta:*5HT, NO and programmed cell death". Acta Biologica Hungarica **55**(1-4), 293-300.

Leise, E. M., K. Thavaradhara, N. R. Durham and B. E. Turner (2001). "Serotonin and nitric oxide regulate metamorphosis in the marine snail *Ilyanassa obsoleta*." American Zoologist **41**: 258-267.

Lin, M.-F. and E. M. Leise (1996). "NADPH-diaphorase activity changes during gangliogenesis and metamorphosis in the gastropod mollusc *Ilyanassa obsoleta*." Journal of Comparative Neurology **374**: 194-203.

Marois, R. and T. J. Carew (1997). "Fine structure of the apical ganglion and its serotonergic cells in the larva of *Aplysia californica*." Biological Bulletin **192**: 388-398.

Meechonkit, P., Kovitvadhi, U., Chatchavalvanich, K., Sretarugsa, P., Weerachatyanukui, W (2010). "Localization of serotonin in neuronal ganglia of the freshwater pearl mussel, *Hyriopsis (Hyriopsis) bialata.*" Journal of Molluscan Studies **76** (3): 267-274.

Meller, E. (2007). "5-HT1A receptor-mediated apoptosis: Death by JNK?". Biochimica et Biophysica Acta, 691–693.

Meyer, J. S., Quenzer, L.F. 2005. "Psychopharmacology: Drugs, the Brain, and Behavior." Sunderland, MA: Sinaur Associates, Inc. 151-160

Miller, S. E. and M. G. Hadfield (1986). "Ontogeny of phototaxis and metamorphic competence in larvae of the nudibranch *Phestilla sibogae* Bergh (Gastropoda: Opisthobranchia)." Journal of Experimental Marine Biology and Ecology **97**: 95-112.

Morse, D. E., H. Duncan, N. Hooker, A. Baloun and G. Young (1980). "GABA induces behavioral and developmental metamorphosis in planktonic molluscan larvae." Federation Proceedings of the Federation of the American Societies for Experimental Biology **39**: 3237-3241.

Morse, D. E., N. Hooker, H. Duncan and L. Jensen (1979). "Gamma-aminobutyric acid, a neurotransmitter, induces planktonic abalone larvae to settle and begin metamorphosis." Science **204**: 407-410.

Olsen, R. W. and W. Sieghart (2009). "GABA(A) receptors: Subtypes provide diversity of function and pharmacology." Neuropharmacology **56**(1): 141-148.

Pechenik, J. A., Cochrane, D. E., Li, W., West, E. T., Pires, A., & Leppo, M. (2007). "Nitric Oxide Inhibits Metamorphosis in Larvae of *Crepidula fornicata*, the Slippershell Snail". Biological Bulletin **213**(2), 160–171.

Peunova, N., B. Kuzin, I. Roberts, C. O'Kane, and G. Enikopolov (1996). "Nitric Oxide, Cell Multiplication, and Cell Survival." Cold Spring Harbor Symposia on Quantitative Biology **61**: 417-426

Pires, A., R. P. Croll and M. G. Hadfield (2000). "Catecholamines modulate metamorphosis in the opisthobranch gastropod *Phestilla sibogae*." Biological Bulletin **198**(3): 319-331.

Richmond, J. E., A. G. M. Bulloch, L. Bauce and K. Lukowiak (1991). "Evidence for the Presence, Synthesis, Immunoreactivity, and Uptake of Gaba in the Nervous-System of the Snail *Helisoma trivolvis*." Journal of Comparative Neurology **307**(1): 131-143.

Romero, M. R., Phuong, M. A., Bishop, C., & Krug, P. J. (2013). "Nitric Oxide signaling deferentially affects habitat choice by two larval morphs of the sea slug *Alderia willowi*: mechanistic insight into evolutionary transitions in dispersal strategies". The Journal of Experimental Biology **216**, 1114–1125.

Ruiz-Jones, G. J. and M. G. Hadfield (2011). "The loss of sensory elements in the apical sensory organ during metamorphosis in the nudibranch *Phestilla sibogae*." Biological Bulletin **220**: 39-46.

Scheltema, R. S. (1962). "Environmental factors affecting length of pelagic larval development in the gastropod, *Nassarius obsoletus*." American Zoologist **2**(3): 445.

Silva, D. M. D., V. P. Ferraz and A. M. Ribeiro (2009). "Improved high-performance liquid chromatographic method for GABA and glutamate determination in regions of the rodent brain." Journal of Neuroscience Methods **177**(2): 289-293.

Sokal, R. R., & Rohlf, F. J. (1995). Biometry: The Principles and Practice of Statistics in Biological Research. New York: W. H. Freeman and Company.

Thavaradhara, K. and E. M. Leise (2001). "Localization of nitric oxide synthase-like immunoreactivity in the developing nervous system of the snail *Ilyanassa obsoleta*." Journal of Neurocytology **30**: 449-456.

Wang, Y., Y. Vodovotz, P. K. M. Kim, R. Zamora and T. R. Billiar (2002). "Mechanisms of Hepatoprotection by Nitric Oxide." Annals of the New York Academy of Sciences **962**: 415-422.

Zhang, Y., He, L.-S., Zhang, G., Xu, Y., Lee, O.-O., Matsumura, K., & Qian, P.-Y. (2012). "The regulatory role of the NO/cGMP signal transduction cascade during larval attachment and metamorphosis of the barnacle *Balanus* (=*Amphibalanus*) *amphitrite*". The Journal of Experimental Biology **215**, 3813–3822.